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(71) Applicant *(for all designated States except US)*: **PROTEIN DESIGN LABS, INC.** [US/US]; 34801 Campus Drive, Fremont, CA 94555 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **VASQUEZ, Maximiliano** [CR/US]; 3813 Louis Road, Palo Alto, CA 94303 (US). **LANDOLFI, Nicholas, F.** [US/US]; 240 Gloria Circle, Menlo Park, CA 94025 (US). **TSURUSHITA, Naoya** [JP/US]; 3719 Redwood Circle, Palo Alto, CA 94306 (US). **QUEEN, Cary, L.** [US/US]; 622 Benvenue Avenue, Los Altos, CA 94022 (US).

(74) Agents: **LIEBESCHUETZ, Joe** et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th floor, San Francisco, CA 94111-3834 (US).

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(54) Title: HUMANIZED ANTIBODIES TO GAMMA-INTERFERON

(57) Abstract: The invention provides humanized immunoglobulins that bind to and neutralize γ-interferon. The antibodies are useful for treatment of diseases of the immune system, particularly autoimmune diseases.

HUMANIZED ANTIBODIES TO GAMMA-INTERFERON

FIELD OF THE INVENTION

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel biologics and, more particularly, for example, to the production of non-immunogenic (in humans) immunoglobulins specific for gamma-interferon (γ-IFN) and their uses in vitro and in vivo. The present invention also relates more specifically to humanized monoclonal antibodies against γ-IFN, polynucleotide sequences encoding the antibodies, a method of producing the antibodies, pharmaceutical compositions comprising the antibodies as an active ingredient, and therapeutic agents for suppressing undesired immune responses comprising the antibodies as an active ingredient.

BACKGROUND

The mammalian immune response is mediated by several types of cells that interact specifically with foreign material, i.e., antigens. One of these cell types, B cells, is responsible for the production of antibodies. Another cell type, T cells, include a wide variety of cellular subsets that destroy cells infected with virus or control the in vivo function of both B cells and other hematopoietic cells, including T cells. A third cell type, macrophages, process and present antigens in conjunction with major histocompatibility complex (MHC) proteins to T cells. Communication between these cell types is mediated in a complex manner by lymphokines, such as interleukins 1-6 and γ-IFN (*see, generally*, Paul, W.E., ed., *Fundamental Immunology, 3rd ed.*, Raven Press, New York (1993), which is incorporated herein in relevant part by reference.)

One important lymphokine is γ-IFN, which is secreted by some T cells. In addition to its anti-viral activity, γ-IFN stimulates natural killer (NK) cells and T helper 1 (Th1) cells, activates macrophages, and stimulates the expression of MHC molecules on the surface of cells (Paul, *op. cit.*, pp. 764-766). Hence γ-IFN generally serves to enhance many aspects of immune function, and is a logical candidate for a therapeutic drug in cases where such enhancement is desired, e.g., in treating cancer. Conversely, in disease states where the immune system is over-active, e.g., autoimmune diseases and organ transplant rejection, antagonists of γ-IFN can be useful to treat the disease by neutralizing the stimulatory effects of γ-IFN.

Mouse monoclonal antibodies that bind to and neutralize γ-IFN have been reported (*see*, e.g., Van der Meide et al., *J. Gen. Virol*, 67, 1059 (1986)). Such anti-γ-IFN antibodies have been reported to delay or prevent rejection in vitro and in vivo mouse models of transplants, (Landolfo et al., *Science* 229, 176 (1985) and Rosenberg et al., *J. Immunol.* 144, 4648 (1990)), both of which are incorporated herein by reference). Treatment of mice prone to develop a syndrome like systemic lupus erythematosus (SLE) with a monoclonal antibody to γ-IFN was reported to delay onset of the disease (Jacob et al., *J. Exp. Med.* 166, 798 (1987)). An anti-γ-IFN antibody has also been reported to alleviate adjuvant arthritis in rats (Jacob et al., *J. Immunol.* 142, 1500 (1989))and colitis in mice. (Powrie et al., *Immunity* 1, 553-562 (1994)). Queen et al., WO 92/11018 discuss the mouse AF2 antibody to γ-IFN, certain humanized immunoglobulins, and use of the same for treating inflammatory disease.

The use of non-human monoclonal antibodies such as AF2 has certain drawbacks in human treatment, particularly in repeated therapeutic regimens as explained below. Mouse monoclonal antibodies, for example, have a relatively short circulating half-life in humans, and lack other important immunoglobulin functional characteristics when used in humans.

Perhaps more importantly, murine monoclonal antibodies contain substantial amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that, after injection of a foreign antibody, the immune response elicited by a patient against the injected antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, if mouse or other antigenic (to humans) monoclonal antibodies are used to treat various human diseases, subsequent treatments with unrelated mouse antibodies may be ineffective or even dangerous in themselves, because of cross-reactivity.

Thus, there is a need for improved forms of humanized immunoglobulins specific for γ-IFN antigen that are substantially non-immunogenic in humans, yet easily and economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

OBJECTS AND SUMMARY OF THE INVENTION

It is the object of the present invention to provide humanized monoclonal antibodies against γ-IFN; polynucleotide sequences encoding the antibodies; a method for producing the antibodies; a pharmaceutical composition comprising the antibodies as an active ingredient; a therapeutic agent for treating diseases, particularly autoimmune diseases, and for immune system suppression comprising the antibody as an active ingredient; and a method for treating such diseases.

The invention provides humanized immunoglobulins that are humanized versions of the mouse AF2 immunoglobulin. The mouse AF2 immunoglobulin is characterized by a light chain variable region designated SEQ ID No:2 and a heavy chain variable region designated SEQ ID No:4. The humanized immunoglobulins of the invention comprise humanized heavy and light chains. Position 11 of the humanized heavy chain variable region framework is occupied by the amino acid present in the equivalent position of the mouse AF2 heavy chain variable region framework. A preferred humanized

immunoglobulin of the invention comprises a humanized light chain variable region designated SEQ ID No:6 and a humanized heavy chain variable region designated SEQ ID No:8.

The humanized immunoglobulins specifically bind to the γ-IFN antigen and neutralize γ-IFN. The humanized immunoglobulins are also capable of blocking the binding of the CDR-donating mouse monoclonal antibody to γ-IFN. -IFN. Preferred humanized immunoglobulins have two pairs of light chain/heavy chain complexes, at least one chain comprising one or more mouse complementarity determining regions (CDRs) functionally joined to human framework region segments. For example, mouse CDRs, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to the antigen at affinity levels stronger than about 10^7 M^{-1}.

The immunoglobulins, including binding fragments and other derivatives thereof, of the present invention can be produced readily by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a first sequence coding for humanized immunoglobulin framework regions and a second sequence coding for the desired immunoglobulin CDRs can be produced synthetically or by combining appropriate cDNA and genomic DNA segments.

The humanized immunoglobulins can be utilized in substantially pure form and can be prepared in a pharmaceutically accepted dosage form, which varies depending on the mode of administration.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A and 1B. Sequences of the cDNA and translated amino acid sequences of the light chain (A) (SEQ ID NOS: 1 and 2) and heavy chain (B) (SEQ ID NOS:3 and 4) variable regions of the mouse antibody AF2. The Kabat CDR sequences are underlined.

Fig. 2A and 2B: cDNA (SEQ ID Nos. 5 & 7) and amino

acid (SEQ ID Nos. 6 & 8) sequences of the mature variable regions of light and heavy chains of humanized antibody HuZAF. Kabat CDRs are underlined.

Fig. 3 Comparison of the heavy chain variable region amino acid sequence of mouse AF2, humanized immunoglobulin HuZAF and humanized immunoglobulins haf25, and HuXAF.

Fig. 4: Neutralization activity of mouse AF2, and humanized antibodies haf25, HuXAF and HuZAF to γ-IFN.

DEFINITIONS

The phrase "substantially identical," in the context of two nucleic acids or polypeptides (e.g., DNAs encoding a humanized immunoglobulin or the amino acid sequence of the humanized immunoglobulin) refers to two or more sequences or subsequences that have at least about 80%, most preferably 90-95% or higher nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using the following sequence comparison method and/or by visual inspection. Such "substantially identical" sequences are typically considered to be homologous. Preferably, the "substantial identity" exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues, or over the full length of the two sequences to be compared. As described below, any two antibody sequences can only be aligned in one way, by using the numbering scheme in Kabat. Therefore, for antibodies, percent identity has a unique and well-defined meaning.

Amino acids from the variable regions of the mature heavy and light chains of immunoglobulins are designated Hx and Lx respectively, where x is a number designating the position of an amino acid according to the scheme of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991). Kabat lists many amino acid sequences for antibodies for each

subgroup, and lists the most commonly occurring amino acid for each residue position in that subgroup to generate a consensus sequence. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. Kabat's scheme is extendible to other antibodies not included in his compendium by aligning the antibody in question with one of the consensus sequences in Kabat by reference to conserved amino acids. The use of the Kabat numbering system readily identifies amino acids at equivalent positions in different antibodies. For example, an amino acid at the L50 position of a human antibody occupies the equivalent position to an amino acid position L50 of a mouse antibody.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. The variable regions of each light/heavy chain pair form the antibody binding site. Thus, an intact antibody has two binding sites.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (*See generally, Fundamental Immunology,* Paul, W., ed., 3rd ed. Raven Press, NY, 1993, SH. 9 (incorporated by reference in its entirety for all purposes)).

From N-terminal to C-terminal, both light and heavy chain variable regions comprise alternating framework and complementarity determining regions (CDRs): FR, CDR. FR, CDR. FR, CDR and FR. The assignment of amino acids to each region

is in accordance with the definitions of Kabat (1987) and (1991), *supra*, and/or Chothia & Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia et al., *Nature* 342:878-883 (1989).

Preferably, analogs of exemplified humanized immunoglobulins differ from exemplified immunoglobulins by conservative amino acid substitutions. For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids may be grouped as follows: Group I (hydrophobic sidechains): met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

The term epitope includes any protein determinant capable of specific binding to an immunoglobulin. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

As used herein, the term "immunoglobulin" refers to tetrameric antibodies as well as a variety of forms besides antibodies; including, for example, Fv, Fab, and $F(ab')_2$ as well as bifunctional hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and single chains (e.g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science 242, 423-426 (1988), which are incorporated herein by reference). (See, generally, Hood et al., Immunology, Benjamin, NY, 2ND ed. (1984), Harlow and Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and Hunkapiller and Hood, Nature, 323, 15-16 (1986), which are incorporated herein by reference.).

As used herein, the term "framework region" refers to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved (i.e., other

than the CDRs) among different immunoglobulins in a single species, as defined by Kabat, et al., op. cit. As used herein, a "human framework region" is a framework region that is substantially identical (about 85% or more) to the framework region of a naturally occurring human antibody.

As used herein, the term "humanized immunoglobulin" refers to an immunoglobulin comprising a human framework, at least one CDR from a non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, i.e., at least about 85-90%, preferably at least 95% identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDRs, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. For example, a humanized immunoglobulin would not encompass a chimeric mouse variable region/human constant region antibody.

The term "patient" includes human and veterinary subjects.

The term "substantially pure" or "isolated" means an object species is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition comprises more than about 80, 90, 95 or 99% percent by weight of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

DETAILED DESCRIPTION

The invention provides humanized immunoglobulins that specifically bind to γ-IFN, and methods of using the same for suppressing undesired immune responses.

I. Humanized Antibodies Specific for γ-IFN

Humanized immunoglobulins of the invention have variable framework regions substantially from a human immunoglobulin (termed an acceptor immunoglobulin), preferably the human acceptor antibody EU, and CDRs substantially from a mouse immunoglobulin termed AF2 (referred to as the donor immunoglobulin). The constant region(s), if present, are also substantially from a human immunoglobulin. The humanized antibodies exhibit a specific binding affinity for γ-IFN of at least 10^7, 10^8, 10^9, or 10^{10} M^{-1}. Usually the upper limit of binding affinity of the humanized antibodies for human γ-IFN is within a factor of 3, 4, 5 or 10 of that of AF2. Often the lower limit of binding affinity is also within a factor of 3, 4, 5 or 10 of that of AF2. Preferred humanized immunoglobulins compete with AF2 for binding to γ-IFN and prevent γ-IFN from binding to and thereby transducing a response through a γ-IFN receptor. The humanized antibodies preferably neutralize 80,90, 95 or 99% of γ-interferon activity at 1, 2, 5, 10, 20, 50 or 100-fold molar excess.

The mouse AF2 antibody is described by Queen et al., WO 92/11018, and has heavy and light chain variable regions designated SEQ ID Nos: 2 and 4. The mouse antibody has IgG2b isotype and a kappa light chain. The heavy and light chain variable regions of the preferred human acceptor antibody EU, and those of other possible human acceptor antibody are described by Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991). The human acceptor antibody is chosen such that its variable regions exhibit a high degree of sequence identity with those of the mouse AF2 antibody. The heavy and light chain variable framework regions can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be consensus sequences of several human antibodies.

The design of humanized immunoglobulins can be carried out as follows. When an amino acid falls under the

following category, the framework amino acid of a human immunoglobulin to be used (acceptor immunoglobulin) is replaced by a framework amino acid from a CDR-providing non-human immunoglobulin (donor immunoglobulin):

(a) the amino acid in the human framework region of the acceptor immunoglobulin is unusual for human immunoglobulins at that position, whereas the corresponding amino acid in the donor immunoglobulin is typical for human immunoglobulins in that position;

(b) the position of the amino acid is immediately adjacent to one of the CDRs; or

(c) the amino acid is capable of interacting with the CDRs (see, Queen et al., op. cit., and Co et al., Proc. Natl. Acad. Sci. USA 88, 2869 (1991), respectively, both of which are incorporated herein by reference). For a detailed description of the production of humanized immunoglobulins see, Queen et al., op. cit., and Co et al., op. cit.

Queen et al., WO 92/11018 report certain humanized forms of AF2, comprising CDR regions from AF2 and variable region frameworks from EU in which certain positions are substituted. The present humanized immunoglobulins preferably contain the same substitutions as described by Queen et al., *supra*. However, additional substitutions are also present. Specifically, position H11 is substituted with the amino acid occupying the equivalent position of the mouse AF2 heavy chain.

Position H11 does not fulfill the criteria for substitution given above, but nevertheless makes a significant contribution to neutralizing activity in humanized immunoglobulins incorporating this substitution. The desirability of substituting at this position was determined

by substitution of various positions in a chimeric AF2 antibody (i.e., having mouse variable domains and human constant regions) with amino acids from equivalent positions in the human EU antibody. Substitution of position H11 caused a significant reduction in the neutralizing activity of the chimeric antibody for γ-IFN.

Usually the CDR regions in humanized antibodies are substantially identical, and more usually, identical to the corresponding CDR regions in the mouse antibody from which they were derived. Although not usually desirable, it is sometimes possible to make one or more conservative amino acid substitutions of CDR residues without appreciably affecting the binding affinity of the resulting humanized immunoglobulin. Occasionally, substitutions of CDR regions can enhance binding affinity.

Other than for the specific amino acid substitutions discussed above, the framework regions of humanized immunoglobulins are usually substantially identical, and more usually, identical to the framework regions of the human antibodies from which they were derived. Of course, many of the amino acids in the framework region make little or no direct contribution to the specificity or affinity of an antibody. Thus, many individual conservative substitutions of framework residues can be tolerated without appreciable change of the specificity or affinity of the resulting humanized immunoglobulin.

Analogs of HuZAF show substantial amino acid sequence identity with HuZAF. Heavy and light chain variable regions of analogs are encoded by nucleic acid sequences that hybridize with the nucleic acids encoding the heavy or light chain variable regions of HuZAF, or degenerate forms thereof, under stringent conditions. Phage-display technology offers powerful techniques for selecting such analogs of HuZAF with retaining binding affinity and specificity (*see, e.g.*, Dower et al., WO 91/17271; McCafferty et al., WO 92/01047; and Huse, WO 92/06204 (each of which is incorporated by reference in its entirety for all purposes).

The variable segments of humanized antibodies produced as described *supra* are typically linked to at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Human constant region DNA sequences can be isolated in accordance with well-known procedures from a variety of human cells, but preferably immortalized B-cells (*see* Kabat et al., *supra*, and WO87/02671). Ordinarily, the antibody contains both light chain and heavy chain constant regions. The heavy chain constant region usually includes CH1, hinge, CH2, CH3, and, sometimes, CH4 regions.

The humanized antibodies include antibodies having all types of constant regions, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. When it is desired that the humanized antibody exhibit cytotoxic activity, the constant domain is usually a complement-fixing constant domain and the class is typically IgG_1. When such cytotoxic activity is not desirable, the constant domain can be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype.

Having conceptually selected the CDR and framework components of humanized immunoglobulins, a variety of methods are available for producing such immunoglobulins. Because of the degeneracy of the genetic code, a variety of nucleic acid sequences encode each immunoglobulin amino acid sequence. The desired nucleic acid sequences can be produced by *de novo* solid-phase DNA synthesis or by PCR mutagenesis of an earlier prepared variant of the desired polynucleotide. All nucleic acids encoding the antibodies described in this application are expressly included in the invention.

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982), which is

incorporated herein by reference). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, NY (1979 and 1981).

II. Therapeutic Methods

Pharmaceutical compositions comprising immunoglobulins of the present invention are useful for parenteral administration, *i.e.*, subcutaneously, intramuscularly and particularly, intravenously. The compositions for parenteral administration commonly comprise a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, histidine and arginine. The concentration of the immunoglobulins in these formulations can vary widely, *i.e.*, from less than about 0.01%, usually at least about 0.1% to as much as 5% by weight and are selected primarily based on fluid volumes, and solubilities in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-100 mg of immunoglobulin. A typical composition for intravenous infusion can be made up to contain 250 ml of sterile Ringer's solution, and 10 mg of immunoglobulin.

Actual methods for preparing parentally administerable compositions are known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science* (15th Ed., Mack Publishing Company, Easton, Pennsylvania, 1980), which is incorporated herein by reference.

The immunoglobulins of this invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilization and reconstitution techniques can be employed. Lyophilization and reconstitution can lead to varying degrees of immunoglobulin activity loss (e.g., with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from an undesired immune response in an amount sufficient to cure or at least partially arrest the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use depend upon the severity of the condition and the general state of the patient's own immune system, but generally range from about 0.01 to about 100 mg of antibody per dose, with dosages of from 0.1 to 50 mg and 1 to 10 mg per patient being more commonly used. Single or multiple administrations on a daily, weekly or monthly schedule can be carried out with dose levels and pattern being selected by the treating physician. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases, in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the present humanized immunoglobulins of this invention, it is possible and may be

felt desirable by the treating physician to administer substantial excesses of these immunoglobulins.

In prophylactic applications, compositions are administered to a patient who is at risk of developing an inappropriate immune response in an amount sufficient to suppress the response. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 0.1 to 100 mg per dose, especially 1 to 10 mg per patient.

The methods are effective on a variety of disease states associated with undesired immune response mediated by HLA class II antigens and/or Th1 cells. Such disease states include graft versus host disease and transplant rejection in patients undergoing an organ transplant, such as heart, lung, kidney, and liver, and autoimmune diseases, such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Hashimoto's thyroiditis, psoriasis primary biliary cirrhosis, and inflammatory bowel disease, e.g., Crohn's disease.

The humanized immunoglobulins can be utilized alone in substantially pure form, or together with a chemotherapeutic agent such as a non-steroidal anti-inflammatory drug, a corticosteroid, or an immunosuppressant. The agents can include non-steroidal anti-inflammatory agents (e.g., aspirin, ibuprofen), steroids (e.g., prednisone) and immunosuppressants (e.g., cyclosporin A, methotrexate cytoxan)

Humanized immunoglobulins of the present invention can also be used in combination with other antibodies, particularly humanized antibodies reactive with other lymphokines or lymphokine receptors. For example, suitable antigens to which a cocktail of humanized immunoglobulins may react include interleukins 1 through 18 and the p55 and p75 chains of the IL-2 receptor (see, Waldmann, *Annu. Rev. Biochem.* 58, 875 (1989) and Queen et al., *Proc. Natl. Acad. Sci. USA* 86, 10029 (1989), both of which are incorporated herein by reference). Other antigens include those on cells responsible for the disease, e.g., the so-called "Clusters of

Differentiation" (Leucocyte Typing III, ed. by A.J. McMichael, Oxford University Press 1987), which is incorporated herein by reference).

Diagnostic Methods

Humanized anti-γ-IFN antibody is also useful in diagnostic methods. Humanized anti-γ-IFN antibody is useful for measuring expression of γ-IFN, and consequent development of an immune response. Methods of diagnosis can be performed in vitro using a cellular sample (*e.g.*, blood sample,lymph node biopsy or tissue) from a patient or can be performed by *in vivo* imaging. Humanized anti-γ-IFN antibody is also useful for purifying human γ-IFN.

In particular embodiments, compositions comprising humanized immunoglobulin of the present invention can be used to detect γ-IFN, for example, by radioimmunoassay or ELISA. Thus, a humanized immunoglobulin of the present invention, such as a humanized immunoglobulin that binds to the antigen determinant identified by the AF2 antibody can be labeled and used to identify anatomic sites that contain significant concentrations of γ-IFN. For examplebut not for limitation, one or more labeling moieties can beattached to the humanized immunoglobulin. Exemplary labeling moieties include, but are not limited to, radiopaque dyes, radiocontrast agents, fluorescent molecules, spin-labeled molecules, enzymes, or other labeling moieties of diagnostic value, particularly in radiologic or magnetic resonance imaging techniques.

The following examples are offered by way of illustration, not by limitation. It will be understood that although the examples pertain to the humanized AF2 antibody, producing humanized antibodies with high binding affinity for the γ-IFN antigen it is also contemplated using CDRs from other monoclonal antibodies that bind to an epitope of γ-IFN.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the constructs, and methodologies that are described in the publications which might be used in

connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

Examples

1. Production of Humanized Immunoglobulins Cloning and sequencing of mouse AF2 variable region cDNAs

Cloning of cDNA sequences encoding the variable regions of the light and heavy chains of the mouse AF2 antibody is described by Queen et al., WO 92/11018. The sequences of these cDNAs are shown in Fig. 1.

Two plasmid vectors were prepared for construction and expression of a chimeric antibody comprising the variable domains of the mouse AF2 antibody linked to human constant regions. The plasmid pVg1-dhfr (Queen et al., supra) contains a human cytomegalovirus IE1 promoter and enhancer (M. Boshart et al., Cell 41, 521 (1985)), the human genomic Cg1 segment including part of the preceding intron, and a dihydrofolate reductase (dhfr) gene (Simonsen et al., Proc. Natl. Acad. Sci. USA 80, 2495 (1984), which is incorporated herein by reference) for selection. The plasmid pVk (Queen et al., supra) is similar to pVg1-dhfr but contains the human genomic Ck segment and the gpt gene. Derivatives of the AF2 heavy and light chain variable regions were prepared from the cDNAs by polymerase chain reaction. The 5' primers hybridized to the V regions starting at the ATG codons and contained XbaI sites; the 3' primers hybridized to the last 15 nucleotides of the J regions and contained splice donor signals and XbaI sites (*see*, Queen et al., *Proc. Natl. Acad. Sci. USA* 86, 10029 (1989), which is incorporated herein by reference). The modified V regions were cloned into the XbaI sites of the respective plasmid vectors between the CMV promoter and the partial introns of the constant regions.

For expression of the chimeric antibody, the heavy

chain and kappa chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones secreting a maximal amount of complete antibody were detected by ELISA. Chimeric AF2 antibody was shown to bind to human γ-IFN by ELISA.

Design of humanized AF2 variable regions

To retain the binding affinity of the mouse antibody in the humanized antibody, the general procedures of Queen et al. were followed (Queen et al. *Proc. Natl. Acad. Sci. USA* 86: 10029 (1989) and U.S. Patent Nos. 5,585,089 and 5,693,762). The choice of framework residues can be critical in retaining high binding affinity. In principle, a framework sequence from any human antibody can serve as the template for CDR grafting; however, it has been demonstrated that straight CDR replacement into such a framework can lead to significant loss of binding affinity to the antigen (Tempest et al., *Biotechnology* 9: 266 (1992); Shalaby et al., *J. Exp. Med.* 17: 217 (1992)). The more homologous a human antibody is to the original murine antibody, the less likely will the human framework introduce distortions into the mouse CDRs that could reduce affinity. Based on a sequence homology search against an antibody sequence database, the human antibody Eu was chosen as providing good framework homology to the mouse AF2 antibody. Other highly homologous human antibody chains would also be suitable to provide the humanized antibody framework, especially kappa light chains from human subgroup I and heavy chains from human subgroup I (as defined by Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed., U.S. Department of Health and Human Services, 1991).

The computer programs ABMOD and ENCAD (Levitt et al., *J. Mol. Biol.* 168: 595 (1983)) were used to construct a molecular model of the AF2 variable domain, which was used to locate the amino acids in the AF2 framework that are close enough to the CDRs to potentially interact with them. To design the humanized HuZAF heavy and light chain variable regions, the CDRs from the mouse AF2 antibody were grafted into the framework regions of the human Eu antibody. At

framework positions where the computer model suggested significant contact with the CDRs, the amino acids from the mouse antibody were substituted for the original human framework amino acids. For the humanized form of AP2 designated HuZAF, this was done at residues 27, 28 (within Chothia CHR H1), 30, 38, 48, 67, 68, 70, 72, 74, 98 and 107 of the heavy chain and at residues 48, 63, and 70 of the light chain. Furthermore, framework residues that occurred only rarely at their positions in the database of human antibodies were replaced by a human consensus amino acid at those positions or by the corresponding mouse antibody amino acids. For HuZAF this was done at residues 93, 95, 98, 107, 108, 109, and 111 of the heavy chain and at residue 48, 63 and 70 of the light chain.

In addition, in HuZAF,position H11 was substituted with the amino acid occupying the equivalent position of the heavy chain of mouse antibody AF2. H11 was identified as being a candidate for substitution by substitution of various positions in a chimeric AF2 antibody (i.e., having mouse variable domains except at substituted positions) with amino acids from equivalent positions in the human EU antibody and testing each variant for reduced neutralizing activity. The final sequences of the HuZAF light and heavy chain variable domains incorporating all of the above substitutions are shown in Figs. 2A and 2B.

Other humanized immunoglobulins were designed also containing mouse AF2 CDR regions and human EU variable regions but containing various subsets of the above substitutions (see Fig. 3). Haf25 is the same as HuZAF except that the antibody lacks substitutions at positions H11 and H38. HuXAF is the same as huZAF except that the former antibody lacks a substitution at position H38.

However, there are many potential CDR-contact residues that are also amenable to substitution and that may still allow the antibody to retain substantial affinity to the antigen. For example, the first four N-terminal amino acid residues in the humanized AF2 light chain can alternatively be substituted with the sequence from the murine antibody because of its contacts with the CDRs.

Likewise, many of the framework residues not in contact with the CDRs in the humanized anti γ-IFN heavy and light chains can accommodate substitutions of amino acids from the corresponding positions of the human EU antibody, from other human antibodies, from the mouse AF2 antibody, or from other mouse antibodies, without significant loss of the affinity or non-immunogenicity of the humanized antibody.

Various alternative amino acids can be selected to produce versions of humanized anti-γ-IFN that have varying combinations of affinity, specificity, non-immunogenicity, ease of manufacture, and other desirable properties. Thus, the examples are offered by way of illustration, not of limitation.

For the construction of genes for the humanized antibodies, nucleotide sequences were selected that encode the protein sequences of the humanized heavy and light chains, plus typical immunoglobulin signal sequences, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restriction sites or to remove undesirable ones. The nucleotide sequences also included the same splice donor signals used in the chimeric genes and an XbaI site at each end. Certain genes were constructed from four overlapping synthetic oligonucleotides. For each variable domain gene, two pairs of overlapping oligonucleotides on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal. The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 bases long with about a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase from each pair of oligonucleotides, digested with restriction enzymes, ligated to the pUC18 vector and sequenced. Two fragments with the respectively correct half-sequences were then ligated into the XbaI sites of the pVg1-dhfr or pVk expression vectors in the appropriate orientations to produce the complete heavy and light chain genes. Certain of the genes for the humanized AF2 variants were generated by PCR mutagenesis of previous genes.

The heavy chain and light chain plasmids were transfected into Sp2/0 mouse myeloma cells by electroporation and cells selected for gpt expression. Clones were screened by assaying human antibody production in the culture supernatant by ELISA, and antibody was purified from the best-producing clones. Antibody was purified by passing tissue culture supernatant over a column of staphylococcal protein A-Sepharose CL-4B (Pharmacia). The bound antibody was eluted with 0.2 M. Glycine-HCl, pH 3.0 and neutralized with 1 M Tris pH 8.0. The buffer was exchanged into PBS by passing over a PD10 column (Pharmacia) or by dialysis.

2. Assay for Neutralizing Activity against γ-IFN

γ-IFN increases the level of expression of MHC molecules on responsive cell lines. Hs294T is a human melanoma cell line that upregulates the amount of MHC class II molecules expressed on the surface when incubated with γ-IFN for 48-72 hr. (Zarniecki et al., J. Immunology, 140, 4217-4223 (1988)). This enhancement can be detected using a monoclonal antibody specific for the upregulated molecule and indirect immunofluorescence and subsequent flow cytometry. An antibody can be assayed for γ-IFN neutralizing activity by measuring whether the antibody inhibits the upregulation of MHC class II molecules on this cell line. γ-IFN for use in the assay was purchased from R&D Systems, 614 McKinley Place, N.E., Minneapolis, MN 55413.

Increasing concentrations of antibody were added to a fixed amount of γ-IFN that had previously been shown to upregulate the level of MHC class II molecules on HS294T cells. The cells were incubated for 48-72 hr with the antibody-γ-IFN mixture and examined for the level of MHC class II molecules by indirect immunofluorescence using a mouse monoclonal antibody specific for human MHC class II molecules. Analysis by flow cytometry allowed for the determination of the median fluorescence intensity of the cell population, which was then plotted against antibody concentration to show the neutralizing capacity of the antibody.

As seen in Fig. 4, HuZAF has significantly better neutralizing activity than haf25, i.e., substitutions at

positions H11 and H38 improved neutralizing activity. HuXAF also had better neutralizing activity than haf25, indicating that substitutions at H11 alone made an important contribution to neutralizing activity.

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What is claimed is

1. A humanized immunoglobulin, which is a humanized version of the mouse AF2 immunoglobulin having a light chain variable region designated SEQ ID No:2 and a heavy chain variable region designated SEQ ID No:4, the humanized immunoglobulin comprising humanized heavy and light chains, provided that position 11 of the humanized heavy chain variable region framework is occupied by the amino acid present in the equivalent position of the mouse AF2 heavy chain variable region framework.

2. The humanized immunoglobulin of claim 1, comprising CDRs from the mouse AF2 immunoglobulin and heavy and light chain variable region frameworks from the human EU immunoglobulin.

3. The humanized immunoglobulin of claim 2, further provided that position H38 is occupied by the amino acid present in the equivalent position of the mouse AF2 heavy chain variable region framework.

4. The humanized immunoglobulin of claim 2, further provided that positions H11, H27, H28, H30, H38, H48, H67, H68, H70, H72, H74, H93, H95, H98, H107, H108, H109, H111 are occupied by the amino acid present in the equivalent position of the mouse AF2 heavy chain, positions L48, and L70 are occupied by the amino acid present in the equivalent position of the mouse AF2 light chain, and position L63 is occupied by the amino acid present in the equivalent position of a consensus sequence of light chains of human immunoglobulins.

5. The humanized immunoglobulin of claim 1 that specifically binds to human γ-IFN with an affinity co..stant within four-fold of the affinity of the mouse AF2 antibody.

6. The humanized immunoglobulin of claim 1 that specifically binds to γ-IFN comprising a humanized mature light

chain having at least 90% sequence identity to the mature light chain of SEQ ID No:6, and a humanized mature heavy chain having at least 90% sequence identity to the mature heavy chain of SEQ ID No:8.

7. The humanized immunoglobulin according to claim 1 that comprises two light chain/heavy chain dimers.

8. The humanized immunoglobulin of claim 1 that is of IgG1 isotype.

9. The humanized immunoglobulin according to claim 1, which is purified to at least 95% homogeneity.

10. A humanized immunoglobulin comprising a mature heavy chain variable region designated SEQ ID No:6 and a mature light chain variable region designated SEQ ID No:8.

11. A pharmaceutical composition comprising a humanized immunoglobulin of claim 1 or 10 and a pharmaceutically acceptable carrier.

12. A method of treating a patient suffering from a harmful immune response, comprising administering a therapeutically effective dosage of the pharmaceutical composition of claim 1 or 10.

13. The method of claim 12, wherein the patient is suffering from an autoimmune disease.

```
ATGGAATCACAGACTCTGGTCTTCATATCCATACTGCTCTGGTTATATGGTGCTGATGGG
 M  E  S  Q  T  L  V  F  I  S  I  L  L  W  L  Y  G  A  D  G

AACATTGTTATGACCCAATCTCCCAAATCCATGTACGTGTCAATAGGAGAGAGGGTCACC
 N  I  V  M  T  Q  S  P  K  S  M  Y  V  S  I  G  E  R  V  T

TTGAGCTGCAAGGCCAGTGAAAATGTGGATACTTATGTATCCTGGTATCAACAGAAACCA
 L  S  C  K  A  S  E  N  V  D  T  Y  V  S  W  Y  Q  Q  K  P

GAGCAGTCTCCTAAACTGCTGATATATGGGGCATCCAACCGGTACACTGGGGTCCCCGAT
 E  Q  S  P  K  L  L  I  Y  G  A  S  N  R  Y  T  G  V  P  D

CGCTTCACGGGCAGTGGATCTGCAACAGATTTCACTCTGACCATCAGCAGTGTGCAGGCT
 R  F  T  G  S  G  S  A  T  D  F  T  L  T  I  S  S  V  Q  A

GAAGACCTTGCAGATTATCACTGTGGACAGAGTTACAACTATCCATTCACGTTCGGCTCG
 E  D  L  A  D  Y  H  C  G  Q  S  Y  N  Y  P  F  T  F  G  S

GGGACAAAGTTGGAAATAAAG
 G  T  K  L  E  I  K
```

FIG. 1A

```
ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCTCTCCCAG
 M  G  W  S  C  I  I  L  F  L  V  A  T  A  T  G  V  L  S  Q

GTCCAACTGCAGCAGCCTGGGGCTGACCTTGTGATGCCTGGGGCTCCAGTGAAGCTGTCC
 V  Q  L  Q  Q  P  G  A  D  L  V  M  P  G  A  P  V  K  L  S

TGCTTGGCTTCTGGCTACATCTTCACCAGCTCCTGGATAAACTGGGTGAAGCAGAGGCCT
 C  L  A  S  G  Y  I  F  T  S  S  W  I  N  W  V  K  Q  R  P

GGACGAGGCCTCGAGTGGATTGGAAGGATTGATCCTTCCGATGGTGAAGTTCACTACAAT
 G  R  G  L  E  W  I  G  R  I  D  P  S  D  G  E  V  H  Y  N

CAAGATTTCAAGGACAAGGCCACACTGACTGTAGACAAATCCTCCAGCACAGCCTACATC
 Q  D  F  K  D  K  A  T  L  T  V  D  K  S  S  S  T  A  Y  I

CAACTCAACAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCTAGAGGATTTCTG
 Q  L  N  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  G  F  L

CCCTGGTTTGCTGACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA
 P  W  F  A  D  W  G  Q  G  T  L  V  T  V  S  A
```

FIG. 1B

```
ATGGAGACCGATACCCTCCTGCTATGGGTCCTCCTGCTATGGGTCCCAGGATCAACCGGA
 M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S  T  G

GATATTCAGATGACCCAGTCTCCGTCGACCCTCTCTGCTAGCGTCGGGGATAGGGTCACC
 D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R  V  T

ATAACCTGCAAGGCCAGTGAAAATGTGGATACTTATGTATCCTGGTATCAGCAGAAGCCA
 I  T  C  K  A  S  E  N  V  D  T  Y  V  S  W  Y  Q  Q  K  P

GGCAAAGCTCCCAAGCTTCTAATTTATGGGGCATCCAACCGGTACACTGGGGTACCTTCA
 G  K  A  P  K  L  L  I  Y  G  A  S  N  R  Y  T  G  V  P  S

CGCTTCAGTGGCAGTGGATCTGGGACCGATTTCACCCTCACAATCAGCTCTCTGCAGCCA
 R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  Q  P

GATGATTTCGCCACTTATTACTGCGGACAGAGTTACAACTATCCATTCACGTTCGGTCAG
 D  D  F  A  T  Y  Y  C  G  Q  S  Y  N  Y  P  F  T  F  G  Q

GGGACCAAGGTGGAGGTCAAACGT
 G  T  K  V  E  V  K  R
```

FIG. 2A

```
ATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGTACCGCGGGCGTGCACTCTCAG
 M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  H  S  Q

GTCCAGCTTGTCCAGTCTGGGGCTGAACTCAAGAAACCTGGGAGCTCCGTGAAGGTCTCC
 V  Q  L  V  Q  S  G  A  E  L  K  K  P  G  S  S  V  K  V  S

TGCAAAGCTTCTGGCTACATCTTTACTAGCTCCTGGATAAACTGGGTAAAGCAGGCCCCT
 C  K  A  S  G  Y  I  F  T  S  S  W  I  N  W  V  K  Q  A  P

GGACAGGGTCTCGAGTGGATTGGAAGGATTGATCCTTCCGATGGTGAAGTTCACTACAAT
 G  Q  G  L  E  W  I  G  R  I  D  P  S  D  G  E  V  H  Y  N

CAAGATTTCAAGGACAAGGCTACACTTACAGTCGACAAATCCACCAATACAGCCTACATG
 Q  D  F  K  D  K  A  T  L  T  V  D  K  S  T  N  T  A  Y  M

GAACTGAGCAGCCTGAGATCAGAGGACACTGCAGTCTATTACTGTGCAAGAGGATTTCTG
 E  L  S  S  L  R  S  E  D  T  A  V  Y  Y  C  A  R  G  F  L

CCCTGGTTTGCTGACTGGGGCCAAGGAACCCTGGTCACAGTCTCCTCAG
 P  W  F  A  D  W  G  Q  G  T  L  V  T  V  S  S
```

FIG. 2B

```
                1                   31    36                  49
huXAF  QVQLVQSGAELKKPGSSVKVSCKASGYIFT sswin WVRQAPGQGLEWIG
huZAF  QVQLVQSGAELKKPGSSVKVSCKASGYIFT sswin WVKQAPGQGLEWIG
DIFF   ---------*-------------------- ----- --*----------
haf25  QVQLVQSGAEVKKPGSSVKVSCKASGYIFT sswin WVRQAPGQGLEWIG
DIFF   ---------------------------- ----- ----------*-

      50                  67                  99          107
huXAF ridpsdgevhymqdfkd KATLTVDKSTNTAYMELSSLRSEDTAVYYCAR gflpwfad WGQGTLVT
huZAF ridpsdgevhymqdfkd KATLTVDKSTNTAYMELSSLRSEDTAVYYCAR gflpwfad WGQGTLVT
DIFF  ----------------- ---------------------------------- -------- --------
haf25 ridpsdgevhymqdfkd KATLTVDKSTNTAYMELSSLRSEDTAVYYCAR gflpwfad WGQGTLVT
DIFF  XXXXXXXXXXXXXXXXX **-*-*-*-*---------------------------------- XXXXXXXX --------
```

FIG. 3

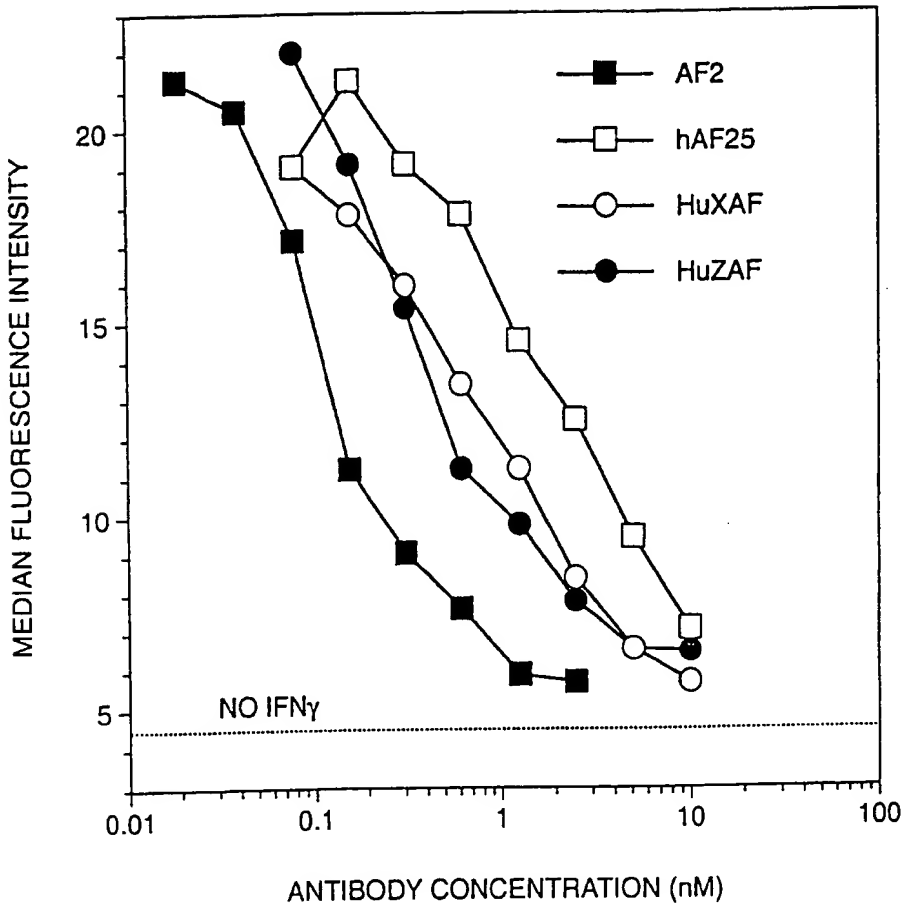
AF2
hAF25
HuXAF
HuZAF
NO IFNγ
MEDIAN FLUORESCENCE INTENSITY
ANTIBODY CONCENTRATION (nM)
20
15
10
5
0.01
0.1
1
10
100

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28195

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 16/00, A61K 39/40, 39/395
US CL : 530/388.1, 424/133.1, 145.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/388.1, 424/133.1, 145.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,530,101 A (QUEEN et al) 25 June 1996 (25.06.1996), column 2, lines 35-67, and column 3, lines 1-58.	1-13
Y	US 5,585,089 A (QUEEN et al) 17 December 1996 (17.12.1996), column 2, lines 37-67, and column 3, lines 1-59	1-13
Y	US 5,693,762 A (QUEEN et al) 02 December 1997 (02.12.1997), column 2, lines 35-67, and column 3, lines 1-59.	1-13
Y	US 5,693,761 A (QUEEN et al) 02 December 1997 (02.12.1997), column 2, lines 37-67, and column 3, lines 1-59.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	07 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Janet Andres Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28195

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim Nos.: 1-13 (in-part)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
no computer-readable sequence was provided; claims were searched to the extent possible

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.